Spatiotemporal Genetic Structure within White Clover Populations in Grazed Swards

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ABSTRACT

White clover (*Trifolium repens* L.) populations exhibit high genetic and clonal diversity, but often exist for many decades in grazed swards at northern midlatitudes. This study was conducted to determine whether genetic structure exists within rapidly changing populations and is a factor in creating genetic diversity. Trifoliate leaf samples were taken monthly in 1997 and 1998 from up to 37 specific points in four 1.2- by 1.2-m quadrats from May to September on two central Pennsylvania farm sites. Random amplified polymorphic DNA (RAPD) profiles of population samples from 67 quadrats were tested by spatial autocorrelation analysis based on a multivariate nonparametric approach. Significant ($P < 0.05$) overall spatial autocorrelation was found in 26 populations that had clones and in seven populations without clones, while nonsignificant autocorrelation was found in 27 and seven populations with and without clones, respectively. Frequencies of significant autocorrelation for populations on one site in 1997 and 1998 were 0.5 and 0.2, respectively, and the frequencies for the other site were 0.8 and 0.4, respectively. The ratio of populations lacking significant autocorrelation to those with significant autocorrelation changed little from zero to six clones, but increased dramatically for seven or eight clones. The estimated patch size (overall mean 66 cm; SE = 8) for two years and two sites did not change significantly over the growing season. Number of clones and patch size was less important in determining genetic structure than variable existence of spatial autocorrelation.

White clover fixes substantial amounts of N, has high nutritional quality as animal feed, and is therefore an important functional component of temperate grazed ecosystems. White clover is an obligately outcrossing tetraploid species, flowers prolifically during the growing season, and produces significant amounts of seed that end up in the viable seed pool (Chapman and Anderson, 1987; Charlton, 1977; Silvertown and Lovett Doust, 1993). Although high viable seed counts have been found in the soil (Chapman and Anderson, 1987; Tracy and Sanderson, 2000), few seeds germinate under field conditions, and few of those seedlings establish as white clover plants (Barrett and Silander, 1992; Brink et al., 1999; Fothergill et al., 1997; Grime et al., 1988).

Chapman and Anderson (1987) showed that buried white clover seed is an ineffective source of new white clover plants in grazed swards and that tap rooted plants survive about 2 yr. Even so, white clover populations often exist for many decades in grazed swards at northern midlatitudes. Presumably this is due to a combination of rare seedling recruitment and clonal growth (Barrett and Silander, 1992; Chapman, 1983; Fothergill et al., 1997; Grime et al., 1988). In addition, somatic mutations during vegetative propagation of clonal plants may produce additional genetic variation (Klekowski, 1984; Slatkin, 1985). Once a plant is established through seedling recruitment, the life span of a tap rooted white clover plant is about 1 to 2 yr (Pederson, 1995). Most of the growth and spreading of plants during the growing season are through stoloniferous propagation (Chapman, 1983), which is the characteristic growth habit of white clover. As more stolon branches are produced, the plant, which is made up of clonal members, expands to cover a greater surface area. Through decay of older stolons and environmental disturbance, a clone frequently fragments into smaller clones. Therefore, physically separate but genetically identical clonal plants may be found in quadrats as small as 1.2 by 1.2 m (Gustine and Sanderson, 2001a,b). Any clone could potentially become a dominant genotype, producing patches covering large areas within grasslands (Cahn and Harper, 1976; Harberd, 1963). Under this scenario, the genetic variability within the white clover population would be reduced, thus increasing the likelihood of a catastrophic loss of plants in a large area from infestation by disease or insects. Spatial structure due to clonal growth can also influence evolutionary processes in white clover by limiting gene flow through breeding of closely related individuals.

Cahn and Harper (1976) did not find the expected low genetic variation nor did they find local domination by one or more clones. The maximum width of clonal patches reported in various field studies is from several centimeters to several meters (Cahn and Harper, 1976; Gustine and Sanderson 2001a; Harberd, 1963). The size of genetic variation among phenotypically distinct clones sampled in a 50-yr-old grassland in North Wales was comparable to that expected between clonal "populations taken from distinctly different environments" (Burdon, 1980). Gustine and Huff (1999) found high genetic variation within and among grazed white clover populations at 18 farms in three northeastern U.S. states using RAPD markers. Widén et al. (1994) surveyed genotypic diversity from data reported in 40 different studies of 45 clonal species and concluded that they were as variable as sexually reproducing plants.

Even at a smaller scale, Gustine and Sanderson (2001a,b) found high genetic variances in 1.8-m^2 quadrats placed in paddocks on Pennsylvania farms. Although white clover populations were genetically variable, RAPD analysis has shown that they nevertheless contain clonal plants (Gustine and Sanderson, 2001a,b), suggesting the presence of genetic structure.

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Abbreviations: RAPD, random amplified polymorphic DNA.

In practice, spatial genetic structure is rarely consistent among loci or within sites. According to Smouse and Peakall (1999), spatial structure is often weaker than expected because gene flow was greater than expected, spatial structure varied with life stages, the statistical procedures used lacked sensitivity, or allozyme markers previously used were less informative than PCR-based genetic markers. Wright (1943, 1946) initially developed the isolation-by-distance model on the basis of the island and continuous models to analyze spatial genetic variation in populations. Spatial autocorrelation analysis can be used to determine if pairs of observations at certain distances apart are more similar (positive autocorrelation) or less similar (negative autocorrelation) than expected for randomly associated pairs of observations (Cliff and Ord, 1981; Sokal and Oden, 1978). The coefficient of spatial autocorrelation most often used in biological applications is Moran's I (Moran, 1950). Recently, Smouse and Peakall (1999) introduced a multivariate approach based on genetic distance methods and nonparametric permutational testing procedures and designed for use with data from PCR-based genetic markers, including RAPDs.

The objective of our study was to determine whether genetic structure exists within rapidly changing white clover populations and whether it is a factor in creating genetic diversity. Answers to such questions could illuminate mechanisms of population growth that make genetically diverse white clover persist over years of grazing. Because white clover spreads and propagates by both vegetative and sexual reproductive means, clonality and isolation-by-distance are confounded in any study designed to separate components of genetic structure created by either reproductive mode. In this report, we examine the population structure of white clover populations using RAPD markers and the spatial autocorrelation analysis method of Smouse and Peakall (1999). We found that many populations studied displayed significant spatial autocorrelation; however, they were equally likely to lack population structure, whether or not clones were detected in the populations.

MATERIALS AND METHODS

The white clover populations used in this study were part of two managed permanent pastures in the ridge and valley physiographic region of Pennsylvania located in Juniata (JU) and Mifflin (MI) counties. These sites were similar in elevation but varied in soil types (Gustine and Sanderson, 2001b). These rotationally stocked swards were not treated with chemical fertilizers. Pastures had been grazed for at least 7 yr since establishment and were never seeded with white clover. White clover populations studied presumably had developed from the soil seed pool (as defined by Silvertown and Lovett Doust, 1993) of naturalized clover. The pastures consisted of mostly white clover (10–60%) and grasses (10–80%), which were composed of tall fescue (*Festuca arundinacea* Schreb.), quackgrass [*Elytrigia repens* (L.) Desv. Ex Nevski], and Kentucky blue grass (*Poa pratensis* L.). The distribution of white clover genotypes was studied in four permanent 1.2- by 1.2-m quadrats on each site and were separated by at least 10 m.

Four quadrats were randomly placed on each of the two farm sites (Gustine and Sanderson, 2001b) in paddocks in early March 1997 before pasture green-up. Each quadrat was divided into a 15- by 15-cm grid pattern to locate 37 predefined sampling points. A trifoliate leaf, if present, was sampled monthly from its undisturbed stolon at those points on each quadrat from April to September as described by Gustine and Sanderson (2001b). We collected 1833 samples, or 62%, of the maximum of 2960 samples that could have been collected during the study (had there been a trifoliate at each sampling point for every quadrat in both years). The total numbers of samples collected in 1997 and 1998 were 957 and 876, respectively. A leaf sample of one to four trifoliates was taken from the same stolon, if present, from each sampling point in a quadrat each month and processed according to Gustine and Sanderson (2001b).

Leaf samples were stored on ice at the time of collection, processed in the lab, and RAPD profiles generated (Gustine and Huff, 1999; Gustine and Sanderson, 2001b). Polymerase chain reactions based on genomic DNA, gel electrophoresis, and ethidium bromide staining was performed according to Gustine and Huff (1999). Gels were documented with a Kodak DC120 digital camera and bands detected with Kodak 1D Image Analysis Software (Eastman Kodak Co., Scientific Imaging Systems, Rochester, NY). Some genomic DNA preparations that did not yield usable RAPD profiles were precipitated in 2 *M* NaCl to remove polysaccharides (Sambrook et al., 1989). Polymerase chain reactions were conducted at least twice on each sample and only repeatable bands were scored.

Three primers (OPA08, OPB14, and OPH12; Operon Technologies, Inc., Alameda, CA) used by Gustine and Huff (1999) were used for this study. Twenty-eight markers were selected that were represented on all of the dates. Marker size was determined by comparison with the 100-bp ladder from Life Technologies (Gaithersburg, MD) and ranged in size from 0.275 to 1.45 kb in length. Markers were scored as present (1) or absent (0). No monomorphic markers were present in the data set. The number of DNA markers per genotype ranged from three to 18.

RAPD profiles of individuals were analyzed by RAPDistance v. 1.04 (Armstrong et al., 1996). The RAPDSTAT module of RAPDistance was used to produce statistics about the populations for each quadrat, listing sample genotypes identical to other samples from the study. We used RAPD profiles to assign genotypes to geographical positions within quadrats. We assumed that those samples with identical RAPD profiles were from the same plant and were thus members of a clone. We also assumed that when multiple trifoliate leaves were combined in a sample from the same stolon, they represented a single clone.

Spatial autocorrelation was analyzed according to the method of Smouse and Peakall (1999) by GenAlEx (Personal communication, Peter E. Smouse, Australian National University, Canberra, Australia; rod.peakall@anu.edu.au). Spatial autocorrelation analysis involves comparing the difference between subjects for some variable of interest—in our case, the difference between their RAPD profiles, called genetic distance—with the difference in the subjects' locations in geographic space. We used the genetic distance measure presented by Huff et al. (1993), which is equivalent to the number of band differences between individuals. Correlograms (Cliff and Ord, 1981) were produced by plotting the spatial autocorrelation coefficients (on the ordinate) calculated at various geographic distance classes (on the abscissa). The distance classes (lags, in geostatistical terminology) are based on arbitrarily chosen intervals into which to group the samples. We examined results using distance class sizes from 22 to 34 cm. Results using a distance class size of 32 cm generally provided the highest overall spatial autocorrelations and will be pre-

sented here. To avoid problems associated with small numbers of sample pairs (Smouse et al., 1986; Upton and Fingleton, 1985), we limited our analysis to quadrats for which we had a minimum of eight pairs of samples each for at least the first four distance classes (0–32, 32–64, 64–96, and 96–128 cm). If the fifth class (128–160 cm) contained fewer than eight pairs of samples, the analysis was conducted with four, rather than five classes. These restrictions resulted in a minimum sample size per quadrat of 11 and a total of 67 out of 80 possible quadrats for analysis. Since the autocorrelation coefficients at each distance class are not independent, significance must be assessed by testing all coefficients simultaneously. Consequently, we report only overall significance, i.e., that of the whole correlogram. The method of Smouse and Peakall (1999) tests significance by comparing the vector of actual autocorrelation coefficients against those derived from a null distribution generated by randomizing the spatial positions of the samples. We used 999 random permutations of the sample positions to generate null distribution.

The point at which the value of the spatial autocorrelation coefficient crosses the abscissa in a correlogram provides an estimate of patch size, or the spatial zone of influence of the variable in question (Fortin, 1999). We extend this definition to mean extent of the area occupied by plants of a clone; however, the geometry of this area is undefined. We calcualted patch size for quadrats with significant ($P < 0.05$) overall spatial autocorrelation by assuming autocorrelation varies linearly between distance classes. The estimated patch size values were pooled over years, sites, and quadrats, and then subjected to one-way ANOVA to test differences among months.

RESULTS AND DISCUSSION

Frequencies of significant ($P < 0.05$) overall spatial autocorrelation for populations on the JU site in 1997 and 1998 were 0.5 and 0.2, respectively, and the frequencies for the MI site in 1997 and 1998 were 0.8 and 0.4, respectively. Thus, frequency of significant overall spatial autocorrelation at both sites in 1998 was about one-half of that in 1997. Figures 1–4 show correlograms for the 67 populations analyzed from the two sites in 1997 and 1998; 33 populations had significant overall spatial autocorrelation coefficients, with 22 in 1997 and 11 in 1998. About 51% of sampled populations (34/67) did not have significant spatial autocorrelation, although 27 of those populations contained one or more clones. That we observed significant overall spatial autocorrelation in some populations showed that white clover genotypes in close proximity were more similar than those with greater physical separation. Each correlogram also reports number of samples, numbers of clones, numbers of nonclonal samples (not a member of a clone), and significance associated with the overall spatial autocorrelation. The number of clones and the number of nonclonal sampled plants detected in each population ranged from zero to eight and three to 33, respectively. Twenty-six populations with clones had significant overall autocorrelations; 27 populations with clones did not have significant overall autocorrelations; seven populations without clones had significant overall autocorrelations; and seven

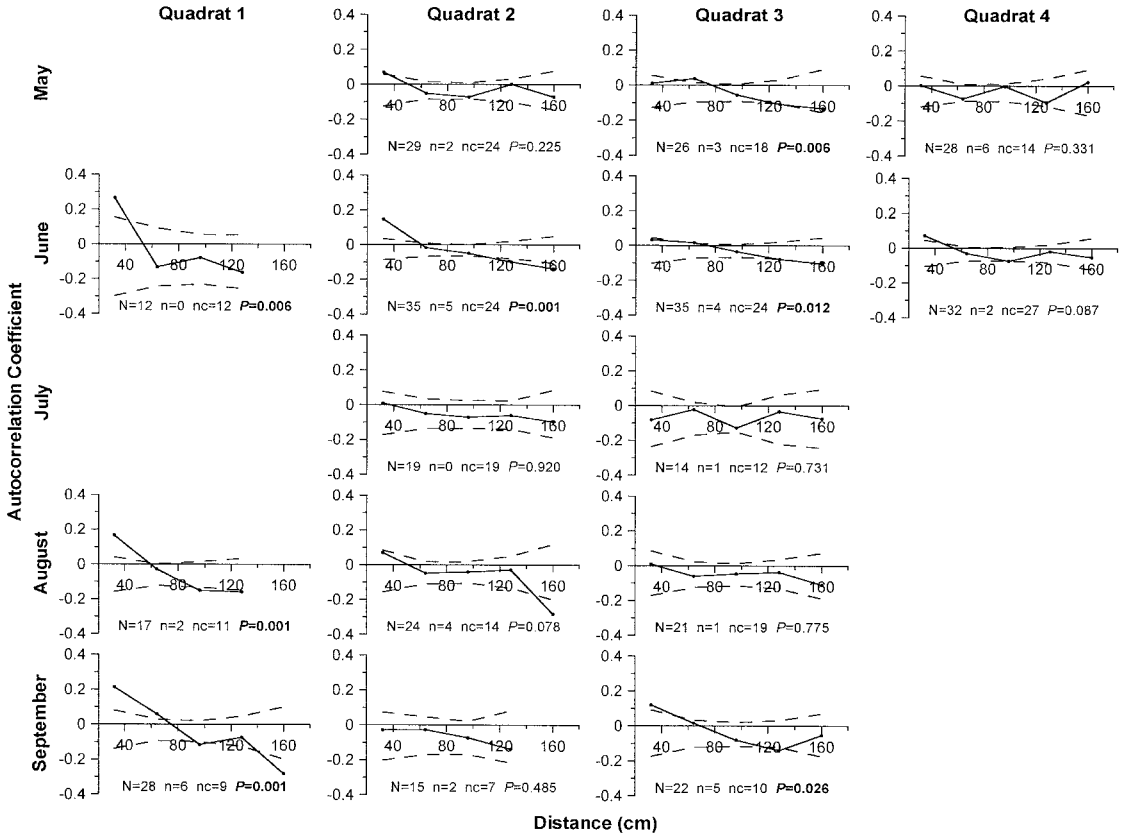

Fig. 1. Spatial autocorrelation correlogram of white clover populations at the JU site in 1997. The dashed lines delineate the 95% confidence intervals of the null distribution. *N*, number of samples; *n*, number of clones; nc, number of nonclonal samples; *P*, overall correlogram significance.

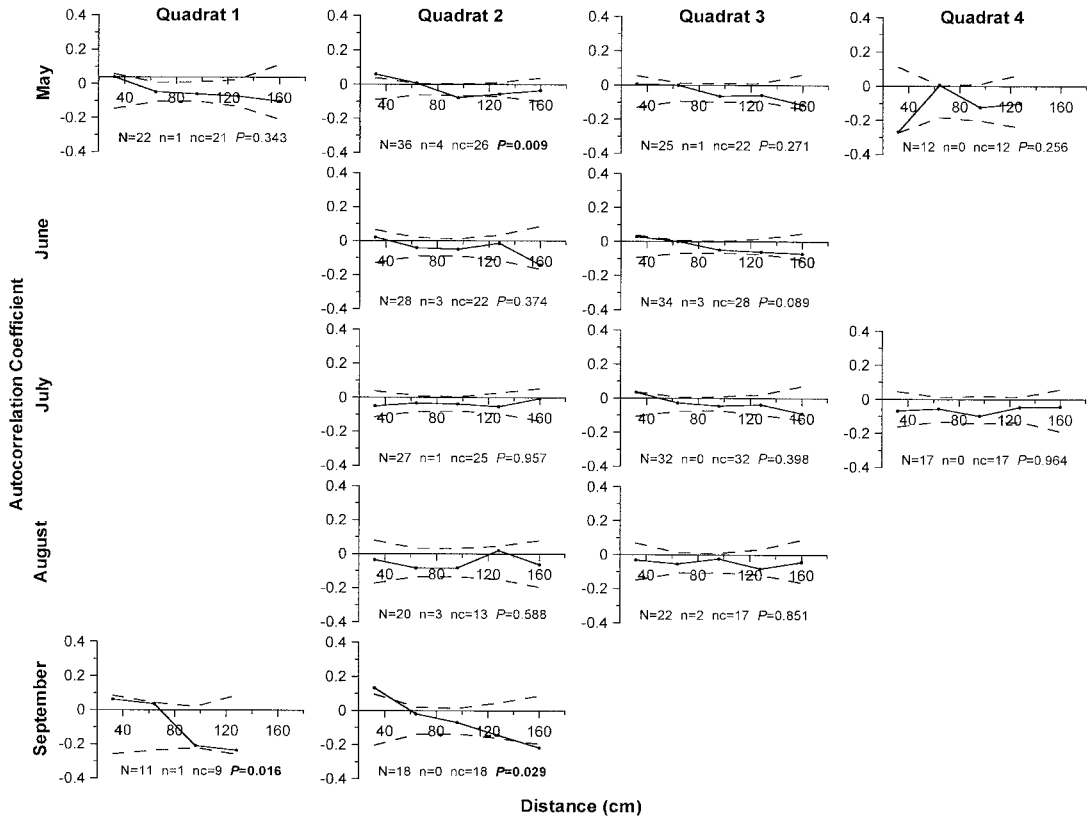

Fig. 2. Spatial autocorrelation correlogram of white clover populations at the JU site in 1998. The dashed lines delineate the 95% confidence intervals of the null distribution. *N*, number of samples; *n*, number of clones; nc, number of nonclonal samples; *P*, overall correlogram significance.

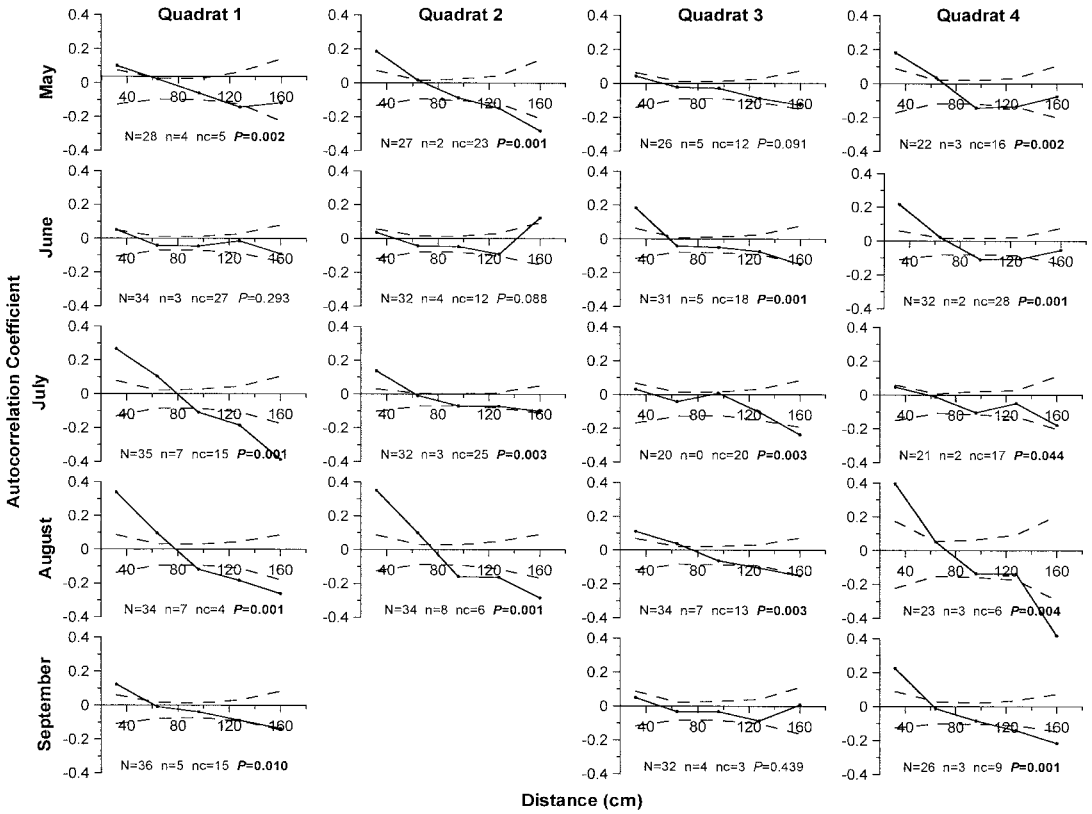

Fig. 3. Spatial autocorrelation correlogram of white clover populations at the MI site in 1997. The dashed lines delineate the 95% confidence intervals of the null distribution. *N*, number of samples; *n*, number of clones; nc, number of nonclonal samples; *P*, overall correlogram significance.

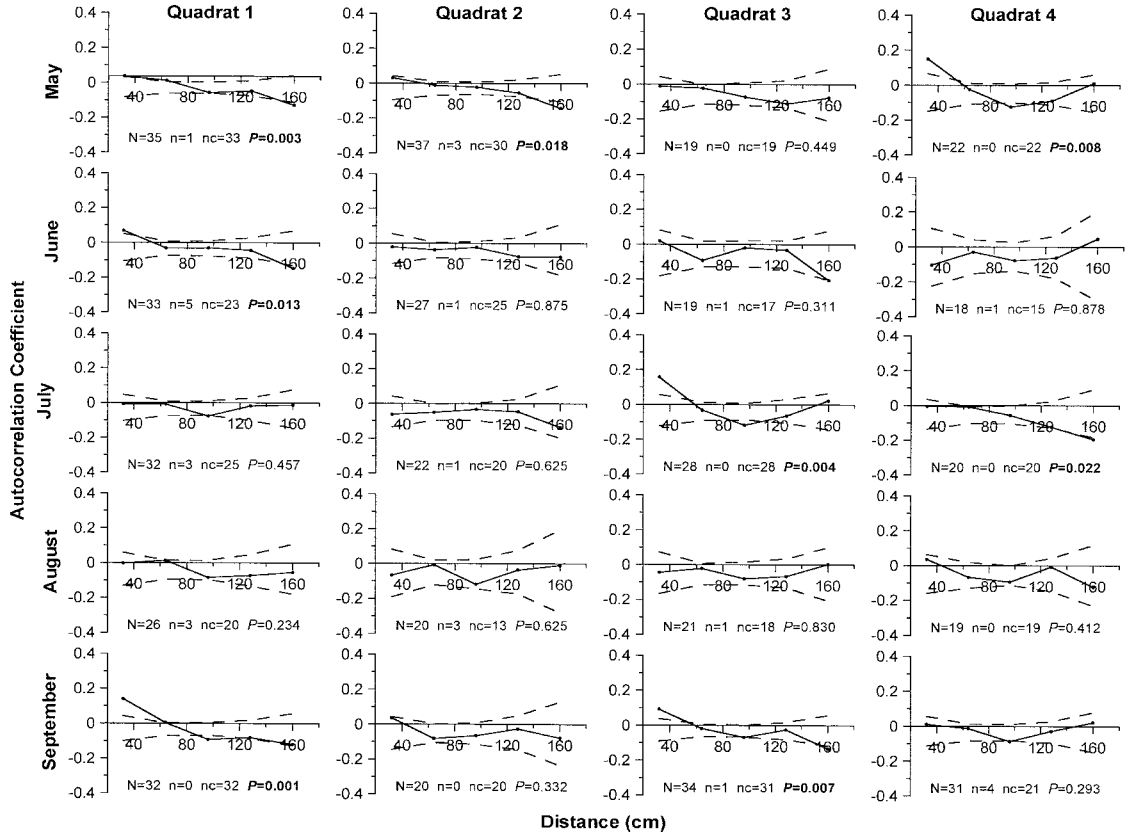

Fig. 4. Spatial autocorrelation correlogram of white clover populations at the MI site in 1998. The dashed lines delineate the 95% confidence intervals of the null distribution. *N*, number of samples; *n*, number of clones; nc, number of nonclonal samples; *P*, overall correlogram significance.

populations without clones did not have significant overall autocorrelations.

When overall significant autocorrelation was not detected in white clover populations with clones, different clonal patches may have been closely related genetically (e.g., siblings) and they may have overlapped sufficiently to break up structure. Therefore, they were not detected as geographically separate patches. The ratio of sampled populations lacking significant overall spatial autocorrelation to those with detectable significant spatial autocorrelation in a quadrat changed little from zero to six clones, but increased dramatically when there were seven or eight clones (four occurrences). Populations in quadrats with seven or eight clones did not show significant spatial autocorrelation, suggesting that the combination of overlapping clones and interspersed plants of different genotypes masked any genetic structure. When there were six or fewer clones, we found that structure was often detected by multivariate spatial autocorrelation.

In 30 instances, the white clover population in a quadrat was analyzed both years in the same month and provided insights into temporal dynamics of spatial autocorrelation. Seven of those quadrats did not show significant spatial autocorrelation either year, while seven other quadrats had significant spatial autocorrelation both years. Twelve of the quadrats had significant spatial autocorrelation in 1997, but not in 1998, whereas four of the quadrats did not have significant spatial autocorrelation in 1999, but did in 1998. Of the 30 instances, significant spatial autocorrelation was found in 19 quadrats in 1997 and in 11 quadrats in 1998. Thus, the number of populations with significant spatial autocorrelation at both sites was less in the second year. Significantly ($P < 0.05$) more clones were found by Gustine and Sanderson (2001b) in1997 on these two sites plus a third site than in 1998.

Estimated patch size did not change significantly ($P > 0.05$) over the growing season (Fig. 5). The mean calculated patch size for both years and both sites was 66 cm (SE = 8) and ranged from 46 to 80 cm. The consistency of patch size over time (Fig. 5) for both sites and years showed that growth for clonal and closely related groups of individuals is limited, probably by environmental pressures typically found in pastures. Research is needed to elucidate the importance of factors such as herbivory by livestock or invertebrates, physical damage by livestock, and competition from more competitive pasture species.

Restricted gene flow, whether in clonal or nonclonal populations, leads to genetic structure in a population as shown by spatial autocorrelation analysis (Bertorelle and Barbujani, 1995; Hartl and Clark, 1989; Mclellan et al., 1997; Smouse and Peakall, 1999; Sokal and Wartenberg, 1983), which makes analyses of spatial patterns useful for examining gene flow in plant populations (Epperson and Allard, 1989; Smouse and Peakall, 1999). In the examination of spatial genetic structure in white clover populations in quadrats at the two sites during

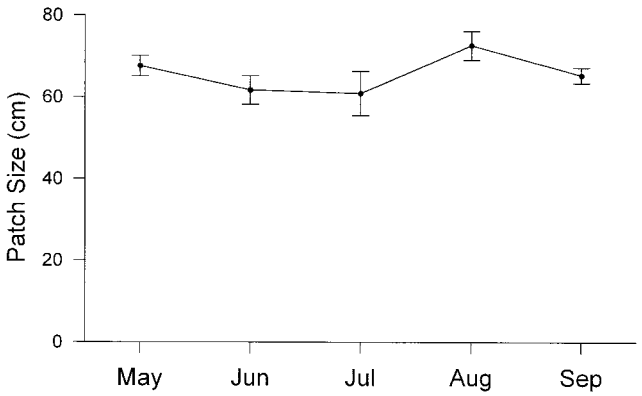

Fig. 5. Mean patch size found in white clover populations averaged over years, sites, and quadrats. Error bars represent ±SE; *n* values for May to September were 8, 6, 5, 5, and 8, respectively.

two consecutive years, we found spatial structure was rarely consistent within loci or sites, in agreement with Smouse and Peakall (1999).

About 10% of the sampled populations (7/67) had significant spatial autocorrelation although no clones were found, i.e., each sampled plant from the quadrat had a different RAPD profile, or genotype. A possible explanation is that a few of the individuals had similar genotypes with only one or two unmatched markers, which placed them in a "family group," and thus they were more similar than genotypes at a greater geographic distance. It is likely that other plants within a quadrat were clonal, but since they were not sampled, they could not be included in the data set.

For genetic structure to be detected, it is necessary for the genetic component to be spatially defined within the population and to be geographically isolated from other genetic groups. Thus, structure will not be evident if there are no distinct clonal patches and random mating has occurred. Lack of genetic structure can occur when multiple clonal patches overlap and intertwine, when there are no clones, or when there is only one genotype in the population. An extreme example of the latter would be if only a single clone is detected in a quadrat and there is only one genotype across the distance classes. Therefore a patch size could not be determined and no genetic structure would be detected. We observed one example of this in a quadrat that was not part of our data set. Thirty-two of 34 sampled plants shared identical RAPD profiles, with the two others interspersed in the quadrat area.

The quadrat dimensions used in this study were appropriate because the calculated patch sizes were always much smaller (60–70 cm; Fig. 5) than the 170-cm maximum distance within the quadrats. Since genetic diversity of white clover populations was high and unchanging at scales ranging from 1.2 by 1.2 m to the North East regional scale (Gustine and Huff, 1999; Gustine and Sanderson, 2001a,b), we expect patch size to be independent of sampling scale. Thus, the patch sizes we found would not change if sampling were scaled up to farm paddock or larger scale. High genetic and clonal diversity has also been reported for other sexually reproducing clonal species (Chung and Epperson, 1999; Ellstrand and Roose, 1987; Harada et al., 1997; Mclellan et al., 1997; Reusch et al., 1999; Sarthou et al., 2001; Stehlik and Holderegger, 2000).

Random amplified polymorphic DNA technology is a reliable method for characterizing variation within and among species and populations (Excoffier et al., 1992; Gustine and Huff, 1999; Huff et al., 1993), although use of RAPDs is not appropriate for some applications, such as parentage analysis where nonparental bands often appear in individual progeny of crosses (Karlovsky, 1990; Perez et al., 1998; Quiros et al., 1995; Riedy et al., 1992; Scott et al., 1992). We think the choice of RAPD markers was appropriate for this study, even though we found about one-third of total markers detected in gels were amplified only once, and therefore counted as absent. This could be construed as a high error rate if we were examining individual plants instead of groups of plants. However, in an unpublished study, Gustine (2000, unpublished) compared RAPD profiles from clonal stolon internodes and trifoliate leaves from four individual plants and found unexplained RAPD marker variation among known clonal genomic DNA preparations. When the data was converted to Euclidian genetic distances with AMOVA (Excoffier et al., 1992) and analyzed by NTSYSpc (Rohlf, 1996) as eight separate groups, the respective stolons and trifoliates of each of the four plants were grouped together at the same genetic distance, but the individual plants were placed at different genetic distances. Thus, a dendrogram of the results revealed a branch for each clone, and each branch corresponded to the stolon and trifoliate sets from the same clonal plant. Since we analyzed groups of plants and not individual plants in the current study, the effects of such errors were minimized. Furthermore, we suggest that most of the markers that amplified only once were likely to represent false positives.

Further evidence of the power of genome markers for tracing current populations is their use to geographically identify progenitor populations and to pinpoint centers of origins for agriculturally important crops species. For example, Heun et al. (1997) used amplified fragment length polymorphism (AFLP) markers to locate the domestication of einkorn wheat (*Triticum monococcum* L.) in the fertile crescent area of the Near East and (Huff et al., 1993) used RAPD genome markers to trace evolution of buffalograss populations in Texas and Mexico. These and the results reported here demonstrate the versatility of population analyses with molecular genomic markers when applied to disparate populations and temporal scales—continental to paddock and centuries to years.

The existence of high genetic and clonal diversity in white clover (Gustine and Sanderson, 2001a,b) coupled with the lack of large dominant clones has puzzled researchers (Burdon, 1980; Cahn and Harper, 1976; Gustine and Huff, 1999). The observed high genetic variability in white clover populations was viewed by Gustine and Huff (1999) to be a result of differential growth of white clover plants driven by changing ecological and environmental factors. Thus, under frequent grazing, defoliated stolons could remain dormant until optimal

growth conditions are restored or they could immediately produce new leaves. Spatiotemporal sampling resulted in a picture of changing genotypes during the growing season. The results reported here are consistent with the notion that at any point in time, only a portion of white clover genotypes in a field will bear leaf samples. At the same time, many individuals without leaves would not be sampled, but would bear leaves later. In this way, white clover populations can maintain high genetic diversity in spite of its clonal growth habit. In addition, rare seedling recruitment adds genetic diversity each year. Therefore, any management schemes imposed by producers in the humid northeastern U.S. that promote white clover growth and maintains about 30% of this species in a grass–legume sward will ensure genetic diversity and persistence of white clover.

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